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**Rie Yanagisawa, Eiko Koike, Tin-Tin Win-Shwe,
Megumi Yamamoto, and Hirohisa Takano**

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Impaired Lipid and Glucose Homeostasis in Hexabromocyclododecane-Exposed Mice Fed a High-Fat Diet

Rie Yanagisawa,¹ Eiko Koike,¹ Tin-Tin Win-Shwe,¹ Megumi Yamamoto,² and Hirohisa Takano³

¹Center for Environmental Health Sciences, National Institute for Environmental Studies, Tsukuba, Japan; ²Department of Basic Medical Sciences, National Institute for Minamata Disease, Minamata, Japan; ³Graduate School of Engineering, Kyoto University, Kyoto, Japan.

Address correspondence to Rie Yanagisawa, Center for Environmental Health Sciences, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba 305-8506, Japan.

Telephone and fax: (81) 29 850 2334. E-mail: yanagisawa.rie@nies.go.jp

Short running title: HBCD enhances high-fat diet-induced obesity

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Abstract

Background: Hexabromocyclododecane (HBCD) is an additive flame retardant used in the textile industry and polystyrene foam manufacturing. Because of its lipophilicity and persistency, HBCD accumulates in adipose tissue and thus has the potential of causing metabolic disorders through disruption of lipid and glucose homeostasis. However, the association between HBCD and obesity remains unclear.

Objectives: We investigated whether exposure to HBCD contributes to initiation and progression of obesity and related metabolic dysfunction in mice fed normal diet (ND)- or high-fat diet (HFD).

Methods: Male C57BL/6J mice were fed HFD (62.2 kcal% fat) or ND and treated orally with HBCD (0, 1.75, 35, and 700 µg/kg body weight) weekly from 6 to 20 weeks old. Body weight, liver weight, biochemical blood tests, histopathological changes, and gene expression profiles in the liver and adipose tissue were examined.

Results: In HFD-fed mice, high- (700 µg/kg) and medium-dose (35 µg/kg) HBCD exposure markedly increased body and liver weight compared with vehicle exposure. This effect was more prominent in the high-dose group. These increases were paralleled by increases in random blood glucose and insulin levels and enhancement of microvesicular steatosis and macrophage accumulation in adipose tissue. HBCD also increased mRNA levels of peroxisome proliferator-activated receptor-γ in the liver and decreased those of glucose transporter 4 in adipose tissue compared with vehicle in HFD-fed mice.

Conclusions: Our findings suggest that HBCD may contribute to enhancement of diet-induced body weight gain and metabolic dysfunction through disruption of lipid and glucose homeostasis, resulting in accelerated progression of obesity.

Introduction

Hexabromocyclododecane (HBCD) (see Supplemental Material, Figure S1) is a brominated flame retardant (BFR) that is incorporated into plastics, electrical and electronic products, textiles, and other materials to decrease flammability (de Wit 2002). HBCD is a ubiquitous and global environmental contaminant. It is highly bioaccumulative because of its persistent and lipophilic properties (Covaci et al. 2006). Human exposure to HBCD occurs mainly through a combination of diet, ingestion of indoor dust, and inhalation of indoor air (Harrad et al. 2010; Roosens et al. 2009). Diet is considered the most important source, particularly in humans consuming large quantities of fish, which contains relatively high levels of HBCD (Janák et al. 2005; Xian et al. 2008). HBCD has been detected not only in human blood and breast milk (Fängström et al. 2008; Weiss et al. 2006) but also in human adipose tissue (Covaci et al. 2008; Malarvannan et al. 2013). Various classes of organohalogenated compounds, including HBCD isomers, were detected in visceral and subcutaneous abdominal fat of obese individuals in Belgium (Malarvannan et al. 2013).

From a toxicological perspective, animal studies have shown that HBCD is a hepatic enzyme inducer (Germer et al. 2006), a developmental neurotoxicant (Eriksson et al. 2006), and an endocrine disruptor (van der Ven et al. 2006). The results of our recent study suggested that some BFRs, including HBCD, activate immune cells and subsequently enhance immune/allergic responses (Koike et al. 2012). Cantón et al. (2008) reported that subacute HBCD exposure downregulates cholesterol biosynthesis- and lipid metabolism-related gene expression in female rat liver, but upregulates drug-metabolizing enzymes such as cytochrome P450 3A in male rat liver. These results suggest that exposure to HBCD may disrupt metabolic, endocrine, and

immune system, resulting in impaired lipid and glucose homeostasis; however, the biological effects of HBCD have not been clarified.

The prevalence of obesity in adults and children has markedly increased worldwide over the last several decades (Ogden et al. 2002). Imbalance between caloric intake and expenditure is considered a key cause of the obesity epidemic, but there is emerging evidence that exposure to environmental chemicals may also be an important contributor (Grün and Blumberg 2007; Newbold 2010). Janesick and Blumberg (2011) recently reported that environmental chemicals such as persistent organic pollutants (POPs) may play an important role in modulating the balance between energy intake and expenditure. Serum levels of POPs such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and organochlorine pesticides may be associated with body mass index, elevated triglyceride levels, abdominal obesity, and cardiovascular diseases (Airaksinen et al. 2011; Ha et al. 2007; Lee et al. 2012; Uemura et al. 2009). In animal studies, POPs including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin, 3,3',4,4'-tetrachlorobiphenyl (PCB-77), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), and POP mixtures in crude salmon oil have been associated with body weight gain, insulin resistance, abdominal obesity, hepatosteatorrhea, and atherosclerosis (Arsenescu et al. 2008; Ruzzin et al. 2010). In the 'obesogen' hypothesis, obesogens are environmental chemicals that promote obesity directly by increasing adipocyte size and/or number, or indirectly by altering metabolic homeostasis or interfering with regulation of appetite and satiety, suggesting that environmental chemicals can regulate lipid metabolism and adipogenesis, and thus promote obesity (Baillie Hamilton 2002; Grün and Blumberg 2007; Newbold 2010). POPs such as TCDD and PCBs have generally been decreasing in the environment and in humans during the last few decades because

of the decline in their use and production (Jones and de Voogt 1999). BFRs including HBCD have globally restricted by the Stockholm Convention on POPs (2010); however, BFRs continue to leach from existing products and to be disposed of in landfills. In addition, the effects of HBCD on obesity and obesity-related metabolic disorders remain poorly understood.

High fat diet (HFD) is a major cause of obesity and is related to development of cardiovascular disease and diabetes. HFD may also contribute to development of systemic inflammation and insulin resistance in these diseases (Grundy 2004; Kahn and Flier 2000). Dietary fat also plays an important role in modulating the metabolism and toxicity of environmental chemicals (Yu 2000). However, the relationship between dietary fat and HBCD has not been examined. In this study, we hypothesized that individuals with diet-induced obesity may be more susceptible to HBCD due to its lipophilic nature and high persistency, leading to metabolic dysfunction via disruption of lipid and glucose homeostasis.

The aim of this study was to determine whether exposure to HBCD induces and/or enhances obesity and metabolic disorders through disruption of lipid and glucose metabolism in the liver and adipose tissue in mice fed normal diet (ND) or HFD.

Materials and Methods

Animals

Five-week-old male C57BL/6J mice were purchased from Japan Clea Co. (Tokyo, Japan) and used for the experiments. Mice were housed individually in polycarbonate cages with wood chip bedding in controlled conditions (12 hr light/dark cycle, 22–26°C, and 40–69% humidity). Food and tap water were provided *ad libitum*. Body weights at the start of the study were 21.4 ± 0.14 g. There were no differences in either group. Then mice were randomly divided into eight groups

(n = 46, 5–6 animals per group), with four groups fed a high-fat diet containing 62.2 kcal% fat (HFD) and the other fed a normal diet (ND; both from Oriental Yeast Co., Ltd., Tokyo, Japan) from the age of 5 weeks (see Supplemental Material, Table S1). HBCD (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) was dissolved in acetone (Nacalai Tesque, Inc., Kyoto, Japan) and diluted with olive oil (Nacalai Tesque). The Ministry of Health, Labour and Welfare (2008) reported that the tolerable daily intake of HBCD is 100 µg/kg body weight (BW)/day (equivalent to a dose of 700 µg/kg BW/week). Therefore, we used four doses of 0, 1.75 (L-HBCD), 35 (M-HBCD), and 700 (H-HBCD) µg/kg BW/week. Mice were dosed vehicle (olive oil containing 0.5% acetone) or HBCD once a week from the age of 6 weeks to 20 weeks, for a total of 15 treatments by oral gavage. Mice were weighed weekly at the time of dosing. Daily water and food intake were monitored in all groups at age of 6, 10, 15, and 20 weeks. All procedures were approved by the Institutional Review Board of the National Institute for Environmental Studies and the National Institute for Minamata Disease. Animals were treated humanely and with regard for alleviation of suffering.

Biochemical tests in serum and analysis of hepatic lipid

All mice were euthanized under diethyl ether anesthesia 24 hr after the final HBCD administration in all groups (5–6 animals per group). The chest and abdominal walls were opened and blood was retrieved by cardiac puncture. Serum was stored at –80°C until use. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Cho), triglyceride (TG), and glucose were measured by SPOTCHEM EZ SP-4430 at 20 weeks of age (ARKRAY, Inc., Kyoto, Japan). Hepatic lipids were extracted by the method of Folch and hepatic T-Cho and TG contents were measured using the enzyme assay by Skylight Biotech, Inc. (Akita, Japan) in four groups (ND+Vehicle, ND+H-HBCD, HFD+Vehicle, and

HFD+H-HBCD group, 5–6 animals per group). Serum insulin levels were measured by ELISA in four groups (Mouse Insulin ELISA KIT; Shibayagi Co., Shibukawa, Japan).

Histopathological examination

Liver and epididymal adipose tissues were fixed in 10% phosphate-buffered formalin (pH 7.4) 24 hr after the final HBCD administration in four groups (ND+Vehicle, ND+H-HBCD, HFD+Vehicle, and HFD+H-HBCD group, 5–6 animals per group). Tissue sections were embedded in paraffin and cut into 4- μ m-thick slices. Liver sections were stained with hematoxylin and eosin. Macrophages in the adipose tissue were detected with anti-mouse F4/80 antibody (Abcam, Cambridge, UK). Adipose tissue sections were treated with inactivated endogenous peroxidase buffer using 3% hydrogen peroxide followed by normal goat serum (5%) for 1 hr at room temperature to decrease nonspecific staining. The sections were incubated in anti-F4/80 antibody (1:1000) overnight at 4°C. Immunohistochemical reactions were performed using the SignalStain Boost IHC Detection Reagent (Cell Signaling Technology, Inc., Danvers, MA, USA) and SignalStain DAB Substrates Kit (Cell Signaling Technology). Slides were counterstained with Mayer's hematoxylin and mounted. The degree of the fatty change in liver and F4/80 positive cells in the adipose tissues was evaluated using an Olympus AX80 microscope in a blinded fashion (Olympus Corp., Tokyo, Japan).

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from liver epididymal adipose tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) in four groups (ND+Vehicle, ND+H-HBCD, HFD+Vehicle, and HFD+H-HBCD group, 5–6 animals per group). Total RNA concentration was assessed spectrophotometrically with a NanoDrop spectrometer (Thermo Scientific, Wilmington, DE,

USA). Total RNA was reverse transcribed to cDNA using a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Foster City, CA, USA). mRNA expression was quantified using the StepOne Plus™ Real-time PCR System (Applied Biosystems). RT-PCR was then performed at 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min, with the last 2 steps repeated for 40 cycles. Data were analyzed by the critical threshold (ΔC_T) and the comparative critical threshold ($\Delta\Delta C_T$) methods using the StepOne Plus™ Software ver. 2.2.2. The relative intensity was normalized to an endogenous control gene (hypoxanthine phosphoribosyltransferase 1; *Hprt1*). TaqMan probes and pairs for target and *Hprt1* genes were listed in Supplemental Material, Table S2.

Glucose and insulin tolerance tests

All mice were fasted for 16 hr or 4 hr prior to performance of a glucose tolerance test (GTT) or insulin tolerance test (ITT), respectively in four groups (ND+Vehicle, ND+H-HBCD, HFD+Vehicle, and HFD+H-HBCD group, 5–6 animals per group). Blood was collected from the tail vein. Mice were injected with D-glucose (2 g/kg p.o.; Wako Pure Chemical Industries, Osaka, Japan) or human insulin (0.75 U/kg i.p.; Sigma-Aldrich) at the age of 17 weeks or 18 weeks, respectively. Blood glucose levels were measured with a blood glucose meter (Glutest Neo Super; Sanwa Kagaku Kenkyusho Co., Nagoya, Japan) at 0, 20, 40, 60, 90, and 120 min. Total values for the area under the curve (AUC; arbitrary units) were obtained without reference to baseline values. Therefore, the area below the observed levels was calculated.

Statistical analysis

Data are expressed as means \pm standard error of mean (SEM). The significance of variation among different groups was determined by one-way ANOVA or Kruskal–Wallis analysis using

the Ekuseru-Toukei 2010 statistical software (Social Survey Research Information Co., Tokyo, Japan). Differences between the experimental and control groups were determined by Dunnett's multiple comparison test or Steel's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

HBCD enhances HFD-induced weight gain and hepatic steatosis

As shown in Supplemental Material, Table S3, an increase in body and liver weight was observed in HFD-fed mice at 20 weeks of age compared with ND-fed mice. In addition, exposure to medium- and high-dose HBCD markedly increased body and liver weight compared with vehicle exposure. In HFD-fed mice, body weight gain by HBCD exposure was observed from 15 weeks of age (see Supplemental Material, Figure S2). In contrast, no alternations in body and liver weight were observed in ND-fed mice with or without HBCD. Food and water intake showed no significant differences between HBCD- and vehicle-exposed groups under both ND and HFD feeding (data not shown). In the histological analysis of the liver, microvesicular steatosis was detected in HFD-fed mice (Figure 1). In addition, H-HBCD exposure in the HFD-fed mice resulted in development of severe microvesicular fatty changes, hepatocyte ballooning, and accumulation of hepatic TG (Figures 1D, E). T-Cho levels in the liver tissues were lower in the HFD+Vehicle group than in the ND+Vehicle group (Figure 1F). Although serum T-Cho levels were higher in HFD-fed mice than in ND-fed mice, HBCD exposure had no significant effect in either group (see Supplemental Material, Table S3). Serum ALT levels were significantly higher in the HFD+M-HBCD and HFD+H-HBCD groups than in the ND+Vehicle group. No remarkable differences in serum TG levels were noted.

Next, to elucidate the mechanisms of HBCD-induced hepatic steatosis in HFD-fed mice, expression of lipid metabolism-related genes in the liver was examined (Figure 1G). In HFD-fed mice, H-HBCD exposure significantly elevated mRNA levels of *Pparg* compared with vehicle exposure ($P < 0.05$). Expression of PPAR γ target genes, such as *Cd36* and *Fabp4* increased in H-HBCD-exposed HFD-fed mice compared with those in vehicle-exposed ND-fed mice. *Fsp27* mRNA was significantly greater in the HFD+H-HBCD group than in the HFD+Vehicle group ($P < 0.05$). H-HBCD induced *Ppara* mRNA in mice fed both diets, but no changes in mRNA levels of *Lxr*, *Rxr*, *Srebp1*, or *Fasn* were observed in either group.

HBCD enhances adipose tissue inflammation in HFD-fed mice

Previous studies have demonstrated that obese adipose tissue is characterized by increased infiltration of macrophages, which may be an important source of inflammation, thereby contributing to the development of metabolic disorders. To evaluate the mechanisms underlying HBCD-induced metabolic abnormality in HFD-fed mice, adipocyte hypertrophy and macrophage infiltration, as revealed by *F4/80* immunostaining in the epididymal adipose tissue, was investigated. No pathological alterations were observed in ND-fed mice in the presence or absence of H-HBCD (Figure 2A, B). HFD feeding accentuated adipocyte hypertrophy and macrophage infiltration (Figure 2C, D). Furthermore, macrophage accumulation in the adipose tissue was more prominent in the HFD+H-HBCD group than in the HFD+vehicle group. Although not statistically significant, HBCD exposure increased mRNA levels of *F4/80* and *Cd11c* (a macrophage marker) ($P < 0.17$ for *F4/80*, $P < 0.17$ for *Cd11c*, Figure 2E). Next, proinflammatory gene expression in adipose tissue was assessed. mRNA levels of *Tnfa* and *Ccl2* in the adipose tissue were higher in HFD-fed mice than in ND-fed mice. *Il1b* and *IL6* mRNA

were higher in the HFD+Vehicle group than in the ND+Vehicle group. H-HBCD exposure elevated *Tnfa* mRNA compared with vehicle exposure, but not statistically significant ($P < 0.12$).

HBCD impairs glucose homeostasis and insulin resistance in HFD-fed mice

Serum glucose and insulin levels were evaluated to determine the impact of HBCD exposure on glucose homeostasis and insulin sensitivity. An increase in random glucose levels was observed with H-HBCD exposure compared with vehicle exposure in both HFD- and ND-fed mice (Figure 3A). Overall, random blood insulin levels were higher in HFD-fed mice than in ND-fed mice, which was more prominent after H-HBCD exposure than after vehicle exposure in the HFD-fed group ($P < 0.05$, vs. HFD+Vehicle group; Figure 3B). Blood glucose levels after 4 or 16 hr of fasting were much higher in HFD-fed mice than in ND-fed mice (Figure 3C, E). In GTT, H-HBCD exposure improved glucose tolerance compared with vehicle exposure in ND-fed mice, whereas similar responses were observed in HFD-fed mice after vehicle and H-HBCD exposure (Figure 3C). No significant differences were found in the total AUC for blood glucose levels in GTT in either group (Figure 3D). In ITT, more resistance to insulin was observed after HBCD exposure than after vehicle exposure in HFD-fed mice (Figure 3E). The total AUC for blood glucose levels in ITT was significantly higher in HFD-fed mice with H-HBCD exposure than in ND-fed mice with vehicle exposure (Figure 3F).

HBCD induces hyperglycemia and hyperinsulinemia via downregulation of glucose transporter in HFD-fed mice

To elucidate the mechanisms of hyperglycemia and hyperinsulinemia following HBCD exposure in HFD-fed obese mice, gene expression of glucose transporter (GLUT) 4 was examined in visceral adipose tissue in RT-PCR analysis (Figure 3G). HFD feeding significantly decreased mRNA levels of *Glut4* compared with ND feeding. *Glut4* expression was much lower in the H-

HBCD group than in the vehicle group under HFD feeding conditions ($P < 0.05$). Next, the effects of HBCD on the insulin-signaling pathways underlying glucose uptake in adipose tissue were elucidated. mRNA levels of *Insr*, *Irs1*, and *Pi3k*, but not of *Akt*, were higher in HFD-fed mice than in ND-fed mice; however, H-HBCD exposure had no apparent effect on mice receiving either diet.

Discussion

In this study, we investigated whether exposure to HBCD induces and/or enhances obesity and obesity-related metabolic dysfunction through disruption of lipid and glucose homeostasis in the liver and adipose tissue in HFD-fed and ND-fed mice. Oral exposure to HBCD was associated with body weight gain, hyperglycemia, hyperinsulinemia, hepatic steatosis, and macrophage accumulation in adipose tissue in HFD-fed mice but not in ND-fed mice. In HFD-fed mice, HBCD also significantly increased *Pparg* mRNA in the liver and decreased *Glut4* mRNA in adipose tissue. These results suggest that HBCD can enhance HFD-induced weight gain and metabolic dysfunction.

This is the first study to report that orally administered HBCD appears to augment effects of high fat diet-induced weight gain and metabolic dysfunction, but it has no effects in mice fed a normal diet. Thus, diet-induced obese individuals may be more susceptible to HBCD compared to lean individuals. Regarding interactions between HFD and environmental chemicals, Wahlang et al. (2013) found that PCB 153 aggravates nonalcoholic fatty liver disease via adipokine dysregulation and altered lipid metabolism in HFD-fed mice but not in control diet fed mice. Hepatic lipid accumulation and inflammation induced by perfluorooctanoic acid (PFOA), a synthetic perfluorinated carboxylic acid, is potentiated by HFD (Tan et al. 2013). These effects

may be due to the highly persistent and lipophilic properties of environmental chemicals in obese individuals, and this greater body burden of HBCD in HFD-induced obesity may enhance weight gain and metabolic dysfunction.

Several studies have suggested that females are more susceptible to HBCD than males. Cantón et al. (2008) reported that subacute exposure to HBCD downregulated cholesterol biosynthesis- and lipid metabolism-related gene expression in female rat liver, but upregulated drug-metabolizing enzyme-related gene expression in male rat liver. Oral exposure to HBCD may induce drug-metabolizing enzymes more prominently in female rats compared to males (Germer et al. 2006). In the present study, HBCD exposure in HFD-fed male mice increased hepatic steatosis and hepatic TG levels (Figure 1D, E), but had no significant effects in ND-fed mice. Thus, the obesogenic effects of HBCD in males may depend on the fat in the diet. Different findings among studies may be also explained by differences in concentration and duration of exposure to HBCD, and further studies are required to define the effects of HBCD, particularly in female mice.

In this study, HBCD in HFD-fed mice elevated the increase in random blood glucose and random blood insulin, and showed a tendency to impair insulin resistance. Furthermore, a significant decrease in mRNA levels of *Glut4* was found in adipose tissue in HFD-fed mice exposed to HBCD. GLUT4 is an insulin-sensitive glucose transporter found in adipose tissue, skeletal muscle, and the heart, and plays a critical role in glucose homeostasis and functions as a key modulator of glucose disposal in fat (Watson et al. 2004). Decreased insulin sensitivity has been reported in GLUT4 null mice (Carvalho et al. 2005). In obesity, decreased GLUT4 gene expression is directly related to development of human insulin resistance (Garvey et al. 1998). Inflammatory molecules generated in adipose tissue, such as TNF- α and IL-6, are related to

decreased GLUT4 expression (Rotter et al. 2003), which leads to insulin resistance due to decreased adipocyte glucose uptake (Leguisamo et al. 2012). In the present study, although not statistically significant, TNF α mRNA increased in the adipose tissue in HBCD-exposed HFD-fed mice. Decreased expression of GLUT4 might, in part, be explained by TNF- α expression. These results suggest that HBCD in HFD-fed mice may decrease glucose transport, resulting in hyperinsulinemia and hyperglycemia. Fat accumulation in the liver and insulin resistance may also enhance metabolic dysfunction and development of hepatic steatosis (Shang et al. 2008). In this study, prominent hepatic steatosis and elevated hepatic TG levels in HFD-fed mice were observed after HBCD exposure compared with vehicle exposure. These findings indicate that HBCD with HFD may have a role in disruption of lipid and glucose metabolism in the liver and adipose tissue.

The current study also showed elevated hepatic mRNA levels of *Pparg* and *Fsp27* in HFD-fed mice exposed to HBCD compared with vehicle. Although there was not statistically significant, similar patterns were observed for target genes of PPAR γ , such as *Cd36* and *Fabp4* (Figure 1G). PPAR γ promotes lipogenesis and adipogenesis in adipose tissue (Wan et al. 2007) and is normally expressed at low levels in human and mouse liver (10–30% of adipose tissue) (Semple et al. 2006). However, HFD induces hepatic PPAR γ expression accompanied by hepatic steatosis (Inoue et al. 2005). Schadinger et al. (2005) showed that PPAR γ in the liver of ob/ob mice (a murine model of type 2 diabetes) induced lipid accumulation in hepatocytes. *Cd36*, *Fabp4*, and *Fsp27* are target genes of PPAR γ involved in fatty acid transportation (Greco et al. 2008) and fat droplet deposition in the liver (Matsusue et al. 2008). CD36 is a membrane receptor associated with uptake of oxidized low-density lipoproteins (Endemann et al. 1993) and mediates hepatic fatty acid uptake, which induces hepatic steatosis (Bradbury 2006). Shearer et al. (2005) found

that FABP4-null mice are protected against diet-induced obesity, insulin resistance, and fatty liver. Fsp27 is a lipid droplet-binding protein that promotes lipid accumulation in adipocytes. A recent study showed that Fsp27 in the liver of ob/ob mice is a direct target gene of PPAR γ and can elevate hepatic TG levels (Matsusue et al. 2008). In contrast, no changes in expression of lipogenesis-related genes such as *Fasn* and *Srebp1* were observed in either diet group in the current study. These results suggest that lipogenesis may not underlie HBCD induced hepatic TG accumulation. Taken together, these results suggest that HBCD exposure in HFD-fed mice activates PPAR γ and, possibly, lipid transport-related genes induced by PPAR γ , and that these changes lead to development of hepatic steatosis.

Chronic and low-grade inflammation are causes of obesity resulting in metabolic dysfunction (Ouchi et al. 2011). Several studies have shown an important role of adipose tissue macrophages in inflammation in obesity through the production and release of proinflammatory mediators such as IL-1, TNF- α , IL-6, and CCL2 (Hrnciar et al. 1999; Xu et al. 2003). IL-1 β and TNF- α are essential for macrophage and adipocyte inflammation. CCL2 plays a critical role in macrophage accumulation and activation. CCL2 expression in adipose tissue induces TNF- α generation through interactions between macrophages and adipocytes (Suganami et al. 2005), thereby blocking insulin signaling in adipocytes (Hotamisligil 2006). In the present study, H-HBCD exposure increased mRNA levels of *F4/80*, *Cd11c*, and *Tnfa*, but not statistically significant, and macrophage accumulation in adipose tissue compared with vehicle exposure in HFD-fed mice (Figure 2E). These findings suggest that HBCD exposure in diet-induced obesity may accelerate adipose tissue inflammation due to increased accumulation of macrophages and, possibly, proinflammatory mediators derived from macrophages.

HBCD dietary intake in humans has been estimated to be 141-151 ng/day in Sweden (Lind et al. 2002) and 350-410 ng/day in the UK (Food Standards Agency 2006). Given the persistent and lipophilic properties of HBCD, there is a need for further investigation of the long-term health effects of chronic low-dose exposure to HBCD *in vivo*. These data may be supportable to demonstrate that HBCD exposure may accelerate the obesity epidemic in human exposure levels.

Conclusions

This study showed that HBCD enhances weight gain, hyperglycemia, hyperinsulinemia, hepatic steatosis, and macrophage accumulation in adipose tissue in HFD-fed mice, but not in ND-fed mice. These results suggest that HBCD may contribute to metabolic dysfunction via an interaction with diet, i.e., HBCD may be an ‘enhancer’ obesogen. This study shows for the first time that HBCD contributes to the progression of diet-induced weight gain and metabolic dysfunction, suggesting that HBCD may increase the risk of diet-induced obesity.

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Figure legends

Figure 1. Histopathological findings and expression of lipid metabolism-related genes in the liver. Histopathological alterations were evaluated using hematoxylin and eosin staining 24 hr after the final HBCD administration. Gene expression in the liver was evaluated using real-time RT-PCR 24 hr after the final HBCD administration. (A) ND+Vehicle group. (B) ND+H-HBCD (700 µg/kg/week) group. (C) HFD+Vehicle group. (D) HBCD+H-HBCD group. (E) Triglyceride contents (mg/g liver). (F) Total cholesterol contents (mg/g liver). (G) mRNA levels in the liver. ND: normal diet, HFD: high-fat diet, TG: triglyceride, T-Cho: total cholesterol. Data were expressed as means \pm SE for 5–6 animals per group. Data were analyzed ANOVA followed by Dunnett's test or Kruskal-Wallis followed by Steel's test. * $P < 0.05$ vs. ND+Vehicle group, ** $P < 0.01$ vs. ND+Vehicle group, # $P < 0.05$ vs. HFD+Vehicle group. Arrows: microvesicular steatosis.

Figure 2. Histopathological findings and gene expression in epididymal adipose tissue. Adipocyte hypertrophy and macrophage accumulation were evaluated using F4/80 immunohistochemical staining 24 hr after the final HBCD administration. Gene expression in adipose tissue was evaluated using real-time RT-PCR 24 hr after the final HBCD administration. (A) ND+Vehicle group. (B) ND+H-HBCD group. (C) HFD+Vehicle group. (D) HBCD+H-HBCD group. (E) mRNA levels in adipose tissue. ND: normal diet, HFD: high-fat diet. Arrows: F4/80-positive cells. Data were expressed as means \pm SE for 5–6 animals per group and analyzed Kruskal-Wallis followed by Steel's test. * $P < 0.05$ vs. ND+Vehicle group, ** $P < 0.01$ vs. ND+Vehicle group.

Figure 3. The impact of HBCD exposure on glucose homeostasis and insulin sensitivity.

Random serum glucose and insulin were measured 24 hr after the final HBCD administration.

GTT and ITT were performed in mice 16 and 4 hr after fasting, respectively. Gene expression of insulin signal-related genes in epididymal adipose tissue was evaluated using real-time RT-PCR

24 hr after the final HBCD administration. (A) Random glucose levels. (B) Random insulin

levels. (C) GTT. (E) ITT. (D, F) Total area under the curve (AUC) for data in C and E,

respectively. (G) Insulin signaling mRNA levels in adipose tissue. Data were expressed as means

\pm SE for 5–6 animals per group. Data were analyzed ANOVA followed by Dunnett's test or

Kruskal-Wallis followed by Steel's test. * $P < 0.05$ vs. ND+Vehicle group, ** $P < 0.01$ vs.

ND+Vehicle group, # $P < 0.05$ vs. HFD+Vehicle group.

Figure 1

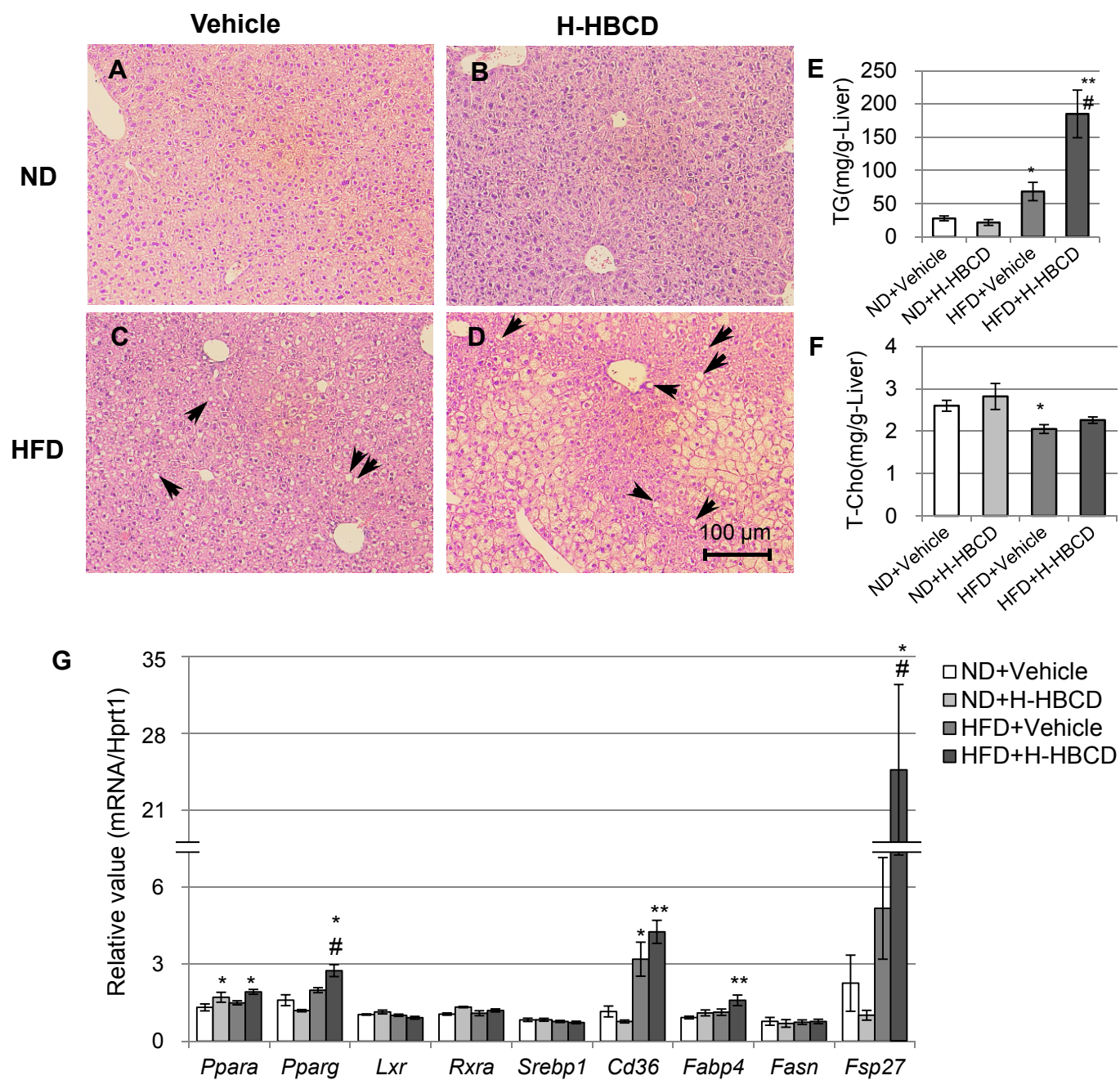


Figure 2

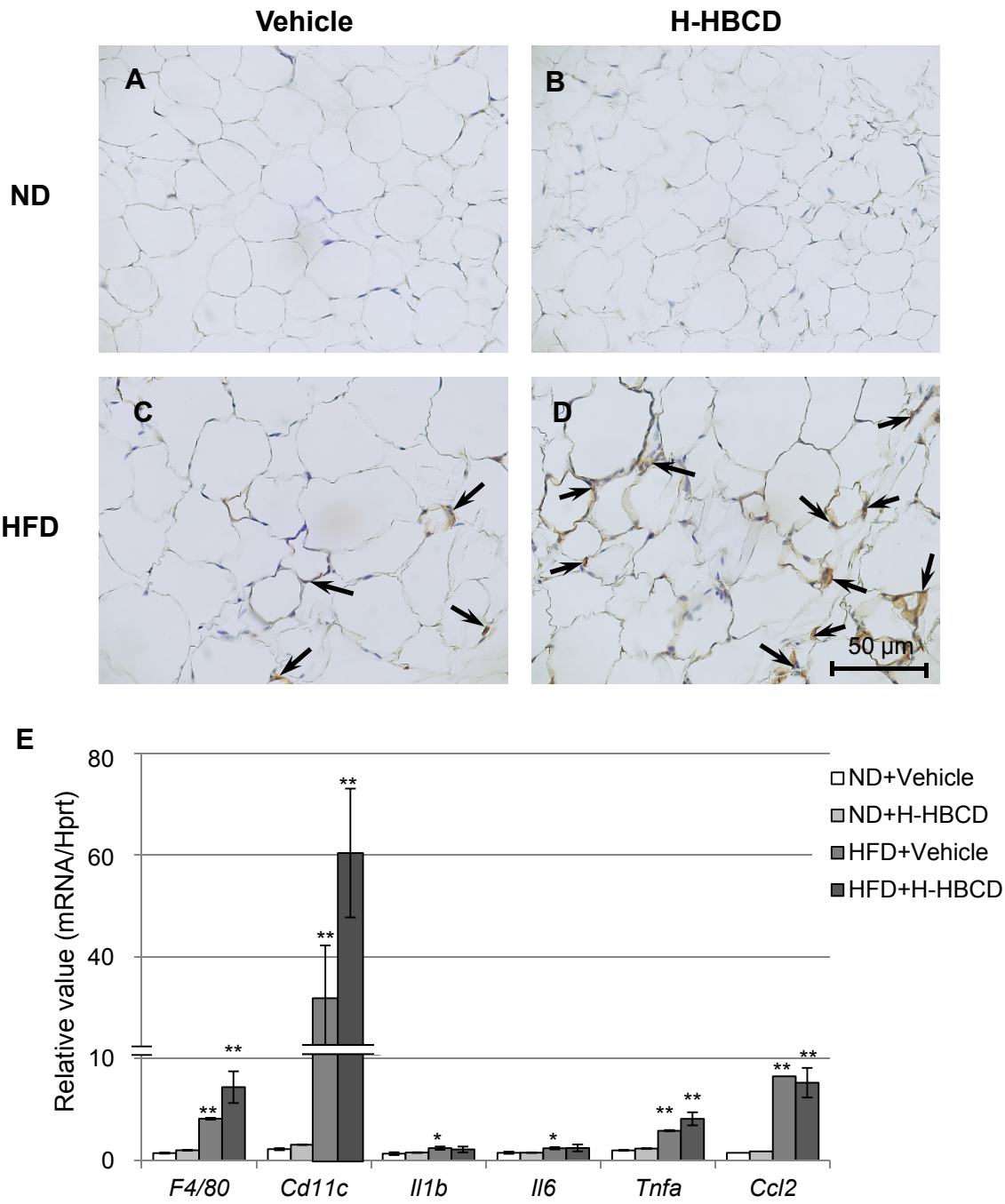


Figure 3

